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14. ABSTRACT
We have a cohort of 200 untreated CFS cases and 400 matched controls that will undergo two novel tests (CyTOF-phosphoflow and HLA Typing through a breakthrough method discovered at Stanford) in order to help enhance our understanding of CFS and contribute to the elucidation of the pathogenesis of the disease.

For CyTOF testing, we are exploring the immune responses by a novel flow cytometer that detects individual cell traits with time-of-flight mass spectrometry (CyTOF). We have completed the CyTOF phenotyping, phospho-flow panel, and gating schemes for testing. Also, the flow cytometry preparation robotics for CyTOF was optimized and antibodies were conjugated. The testing is ongoing and we hope to continue testing as permitted.

For HLA typing testing, we are determining the human leukocyte antigens (HLA) types using a novel method that combines long-range polymerase chain reaction (PCR) with very high-throughput, multiplexed Illumina paired-end sequencing of genes permitting direct haplotyping. We have identified the DNA PAXgene tubes that will be used for testing. Also, we have identified the best possible conditions to amplify all the HLA gene of interest, namely for the class I genes HLA-A, -B, -C and for the class II genes: HLA DQA, DQB, DRB DPA and DPB. Lastly, we achieved the goal of robust and highly reproducible amplification of each

In summary, for CyTOF testing we finalized the Phospho-CyTOF panel and have begun testing samples. To date we have tested 54 samples. These samples will continue to be tested for Year 2. The data is being received; no analysis has been done yet. The data will be analyzed by our statistical team. For HLA typing testing, all the DNA PAXgenes have been identified and separated into different batches. To date, two plates have been run and are currently being analyzed. To date we have tested 180 samples Year 1 has allowed us to ready the logistics and to begin CyTOF and HLA Typing testing.

15. SUBJECT TERMS
CyTOF, human leukocyte antigens (HLA) types, Chronic Fatigue Syndrome(CFS), novel testing, pathogenesis, phenotyping, phospho-flow, flow cytometry, antibodies, haplotyping

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INTRODUCTION:

We have a cohort of 200 untreated CFS cases and 400 matched controls that were enrolled March 2, 2010 - September 1, 2011. The funds for recruitment of this cohort were financed by a separate gift fund. With the help of funding from the DOD grant, we are able to conduct two novel tests (CyTOF-phosphoflow and HLA Typing) in order to help learn more about CFS and contribute to the elucidation of the pathogenesis of the disease. We are analyzing the existing blood samples that have already been collected and are testing the samples within each respective 'matched set' that are matched by age and gender. A matched set includes a CFS case sample with two corresponding matched controls. We feel that Year 1 has allowed us to ready the logistics for CyTOF and HLA Typing testing and look forward to continue the testing in Year 2. Both Tests are being done concurrently under the supervision of Dr. Holden Maecker (CyTOF) and Dr. Michael Mindrinos (HLA Typing) from the Stanford Human Immune Monitoring Center and Department of Genetics, respectively.

BODY (CyTOF):

We are exploring the immune responses by a novel flow cytometer that detects individual cell traits with time-of-flight mass spectrometry (CyTOF). CyTOF will allow, for the first time, a single cell-based as well as panoramic view of immune cells and their signaling networks in CFS patients. We have completed the CyTOF phenotyping, phospho-flow panel, and gating schemes for testing. Testing is ongoing and we hope to continue testing as permitted.

The CyTOF technician, Dr. Rosemary Fernandez, has validated the CyTOF phenotyping and phospho-flow panel. From September 2012 - March 2013, the workflow for testing was optimized by Dr. Fernandez. She tested available antibody combinations and finalized the panel to be used. All stimulation conditions, cell subsets, and phospoepitopes were decided upon and approved. The flow cytometry preparation robotics for CyTOF were optimized, a process that included titration of antibodies and adjustment of washing steps, with repeated comparisons to manual processing. Also, the antibodies needed for testing were conjugated, and a large batch of reagents was created and tested, to minimize the need for later batch-to-batch validation. Repeated runs of the same replicate sample on this platform were performed to ensure reproducibility.

<u>The basic steps of the CyTOF phospho-flow assay include:</u>

- Thaw cells, wash, count
- Rest at 37oC
- Stimulate with cytokines
- Fix cells
- Wash
- Surface stain for 30 min

- Wash
- Permeabilize cells overnight in Methanol at 0C
- Wash
- Intracellular staining for 30 min
- Wash
- Intercalator (Ir) staining for 20 min and wash x 2 with MilliQ water and run on CyTOF

We began running the peripheral blood mononuclear cell (PBMC) samples from CFS patients and matched controls from March 2013 to the present. As of June 10, 2013, a total of 54 patient and control samples have been tested. Each patient sample has 8 stimulation conditions, so this represents a total of 432 tubes run on the CyTOF. An internal control is being tested and used to check for variability between batches. There are enough frozen aliquots from the same internal control to last the duration of the study. Preliminary gating scheme of the data from these samples has been done (See Appendix A), and an interim analysis by the statistical team is planned. Visual inspection of the data suggests that there is reasonable reproducibility of the internal control sample between batches, and possible differences in some of the test samples. However, we remain blinded to the identity of patients versus controls, so as not to bias the analysis.

BODY (HLA Typing):

We are determining the human leukocyte antigens (HLA) types using a novel method that combines long-range polymerase chain reaction (PCR) with very high-throughput, multiplexed Illumina paired-end sequencing of genes permitting direct haplotyping. To this end we are ongoing with testing the samples for HLA typing. Once we complete testing, we will assess whether HLA profiles influence the role that specific pathogens may be playing in the development of CFS.

We began by finding all of the DNA PAXgene tubes for the 600 samples to be tested. 596 DNA PAXgenes were found, 4 samples were not found because no DNA PAXgene tubes were collected for these patients, although RNA PAXgenes were. We will either have to replace these 4 samples or only test 596 out of the 600 samples. We separated the DNA PAXgene tubes into 7 different batches of about 90 samples per batch. These batches were given for DNA isolation. Each batch comprises one plate containing approximately 90 patient samples. The isolated DNA plates contained approximately 6 microliters per sample. The plates used are 96-well micro-titer plates that are well defined and annotated with an electronic map (See Appendix B).

To start, two batches were extracted to create two plates containing 90 samples each (2plates=180 samples). The first two batches for isolation were in December 2012, and Dr. Mindrinos began testing for

plates 1 and 2 around February/March 2013. The next batches, 3 & 4, were isolated in March 2013 and given to Dr. Mindrinos June 2013. Currently for batches 5 & 6, the DNA isolation is being conducted.

At the Stanford Genome Technology Center (SGTC) we have identified the best possible conditions to amplify all the HLA gene of interest, namely for the class I genes HLA-A, -B, -C and for the class II genes: HLA DQA, DQB, DRB DPA and DPB. We achieved our goal of robust and highly reproducible amplification of each gene by either selecting one or two set of long range PCR primers to amplify the regions of interest for each locus. For the HLA -A, -B, -C, DRB, and DPA and DPB we have optimized the PCR conditions by using only one set of PCR primers to amplify all the polymorphic exon of interest while in the case of HLA -QA and –QB they have designed two overlapping set of primes, with each one amplifying an amplicon of approximately 4 to 4.5 kb.

At this current stage of the technology all long range PCRs for each subject are carried out individually for each gene and all the products for each subject are pooled for preparation for the Illumina sequencing. The sample preparation protocol starts with mixing the DNA of each subject with the long range PCR primers and the PCR reaction mix using a bench top robotic instrument capable of processing 96 samples at a time. For each sample eight individual reactions are prepared, one for each of the major class I or class II HLA genes, in the pre PCR room to prevent sample contamination.

After pooling the PCR products from each subject together using the Biomek® FX instrument (See Appendix C) in a 96 micro-titer plate they randomly sheared them to an approximately optimum size ranging from (700-200bp) using the Covaris Acoustic Sonicator. After the sonication the micro-titer plate is transferred back to the Biomek® FX to continuous with the addition of home-made optimized barcodes, to differentiate the different subjects in the sequencing run, and the appropriate Illumina sequences are incorporated to facilitate the library preparation for sequencing. To optimize the efficiency and effectiveness of the library preparation equimolar amounts of each subject we have incorporated a qPCR step in order to pool almost equimolar amounts of each subject before sequencing.

The first two micro-titer plates have been tested by Dr. Mindrinos and on July 2013 Dr. Mindrinos was working on analyzing the data for the 1st 2 plates. We have done DNA isolation for 360 study samples that Dr. Mindrinos has for HLA typing testing. To date, 180 patients have been tested and 180 more patient samples are ready for testing.

KEY RESEARCH ACCOMPLISHMENTS:

- Year 1 has allowed us to ready the logistics and to begin CyTOF and HLA Typing testing

For CyTOF, there have been 54 samples tested.

- Completed the CyTOF phenotyping, phospho-flow panel, and gating schemes for testing
- Validated the CyTOF phenotyping and phospho-flow panel
- All stimulation conditions, cell subsets, and phospoepitopes were decided and approved
- Flow cytometry preparation robotics for CyTOF were optimized
- Antibodies needed for testing were conjugated
- Large batch of reagents were created and tested
- Began running PBMC samples from CFS patients and matched controls in March 2013
- Preliminary gating scheme of the data from these samples has been done

For HLA Typing, there have been 180 samples tested.

- Identified the DNA PAXgene tubes that will be tested
- Identified the best possible conditions to amplify all the HLA gene of interest
- Achieved goal of robust and highly reproducible amplification of each gene
- Optimized the PCR conditions for the HLA -A, -B, -C, DRB, and DPA and DPB
- Incorporated qPCR step to pool almost equimolar amounts of each subject before sequencing.
- The first two micro-titer plates have been tested
- Work is being done to analyze the data for the 1st two micro-titer plates.

REPORTABLE OUTCOMES:

There are no current reportable outcomes as we are still in the testing phase.

CONCLUSIONS:

This research will help validate the model of CFS as a heterogeneous illness that results from a complex interaction of genetic, immune, and infectious factors. Characterizing such traits within CFS populations may not only assist in distinguishing CFS subjects but also allow formation of CFS subgroups. In the long term, the ability to discern CFS subgroups may lead to generation of diagnostic tests and effective targeted therapies.

Our CFS team has scheduled weekly team meetings where action items and any updates on the status for the DOD grant are discussed. There are ad hoc meetings as needed to discuss the budget, testing, and timelines with the collaborating members, Dr. Maecker and Dr. Mindrinos, as well.

In summary, for CyTOF testing we have finalized the Phospho-CyTOF assay and have begun testing samples. These samples will continue to be tested for Year 2. Data is being received and filed. For HLA typing testing, all the DNA samples have been identified and separated into different batches. Each batch will be given for DNA isolation and a plate of approximately 90 patient samples will be created. To date, two plates have been run and are currently being analyzed.

Overall, a lot has been done to get the testing in place. We look forward to the continuation of the testing phase of these samples in Year 2.

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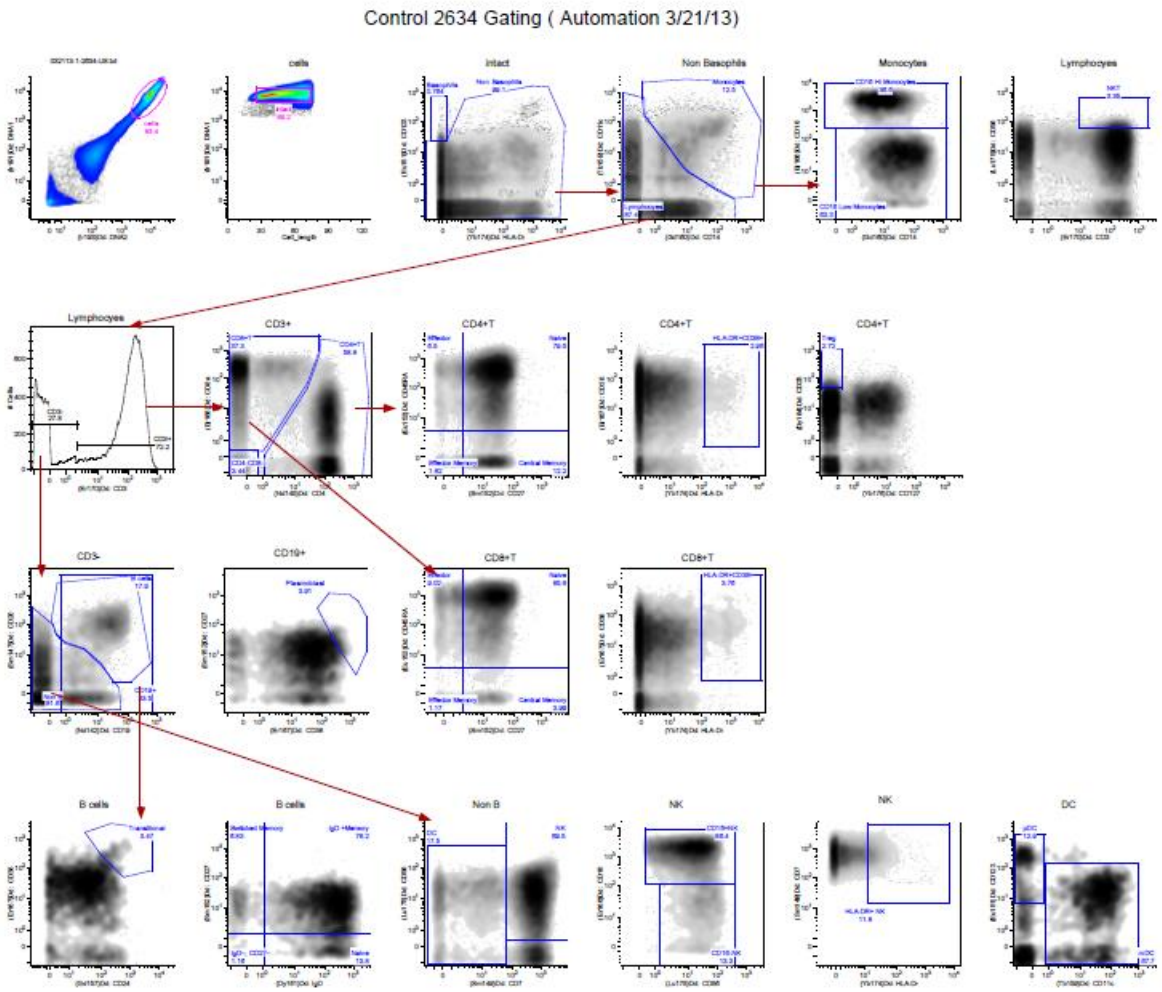
APPENDICES:

Appendix A: Mapping of internal control gating scheme

Appendix B: Electronic map of isolated DNA plate for HLA Typing testing

Appendix C: Photo of Biomek® FX instrument

Appendix A: Mapping of internal control gating scheme



Appendix B: Electronic map of isolated DNA plate for HLA Typing testing

gDNA plate#1 for JM, 2/5/2013

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 1507	2 1161	3 981	4 890	5 876	6 537	7 1878	8 402	9 1260	10 1089	11 925	12 704
B	13 1106	14 1584	15 658	16 1408	17 871	18 2661(1055)	19 823	20 857	21 1153	22 280	23 1703	24 739
C	25 1246	26 891	27 564	28 937	29 357	30 426	31 1116 (1396)	32 962	33 549	34 930	35 638	blank
D	36 1228	37 1663	38 583	39 1236	40 1265	41 191	42 348	43 1076	44 1674	45 914	46 881	blank
E	47 1575	48 928	49 555	50 3	51 25	52 1337	53 902	54 904	55 868	56 21	57 946	blank
F	58 1688	59 983	60 1316	61 57	62 935	63 1147	64 1056	65 905	66 993	67 385	68 1462	blank
G	69 969	70 874	71 1825	72 260	73 1086	74 1117	75 922	76 506	77 1392	78 1885	79 1954	blank
H	80 851	81 1261	82 1781	83 1001	84 1845	85 1437	86 998	87 405	88 1004	89 915	90 213	blank

Appendix C: Photo of Biomek® FX instrument

http://www.labwrench.com/?equipment.view/equipmentNo/3090/Beckman-Coulter/Biomek-FX/